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Synthesis of calbindin-D28K during mineralization in human bone marrow stromal cells

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1α,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is known to modulate Ca^{2+} metabolism in several cell types. Vitamin-D-dependent calcium binding proteins such as calbindin-D28K (28 kDa calcium binding proteins) have been shown to be regulated by 1,25$(OH)_2D_3$ but the mechanisms controlling calbindin synthesis are still poorly understood in human osteoblast cell culture models. The human bone marrow stromal cells (HBMSC) described in this paper developed a calcified matrix, expressed osteocalcin (OC), osteopontin (OP) and responded to 1,25$(OH)_2D_3$. The expression of vitamin D receptor mRNA was demonstrated by reverse transcription-PCR. Calbindin-D28K protein was identified only in cells arising from the sixth subculture, which exhibited a calcified matrix and all of the osteoblastic markers, e.g. OC and OP. It was demonstrated by dot-immunodetection using immunological probes, and by *in situ* hybridization using labelled cDNA probes. Moreover, vitamin D_3 enhanced calbindin-D28K synthesis as well as OC synthesis and alkaline phosphatase activity. Uptake of ^{45}Ca induced into the matrix by 1,25$(OH)_2D_3$ supports the hypothesis that the calcium-enriched matrix could trap calbindin-D proteins. In conclusion, the studies *in vitro* described in the present paper indicate, for the first time, a possible role of calbindin-D28K in mineralized matrix formation in HBMSC.

INTRODUCTION

Bone formation is the co-ordinate function of osteoblasts. *In vitro*, mineralization of human osteoblast-like cultures is associated with the expression of mature bone-specific markers such as osteopontin (OP) and osteocalcin (OC), matrix proteins regulated in part by vitamin D_3 [1]. The functional metabolism of vitamin D_3 consists of an initial 25-hydroxylation step, primarily in the liver, followed by a 1-hydroxylation of 25-hydroxyvitamin D_3 in the kidney. The active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], has been shown to regulate mineral homoeostasis and exerts a key role on bone growth [2].

Physiological responses to vitamin D_3 are regulated by both non-genomic [3] and genomic events [4]. The genomic mechanisms of the hormone are mediated by the vitamin D receptor (VDR), an intracellular receptor that is part of the steroid hormone superfamily of receptors [2]. The presence of VDRs has been described not only in typical target organs for 1,25$(OH)_2D_3$ such as intestine, kidney and bone tissues but also in cell lines not directly related to mineral metabolism [5]. The 1,25$(OH)_2D_3$ receptors have been well characterized in osteoblast-like cells such as ROS 17/2.8 [6], MC3T3 [7], rat osteoblast-like cells [8] and more recently in murine bone marrow stromal cells [9]. Although the human VDR has been cloned [10], the functional receptor remains unidentified in human bone cells or human bone marrow stromal cells (HBMSC).

It is recognized that steroid hormones support osteoblast proliferation, differentiation and mineralization [4]. Osteoblast differentiation follows a sequential process with stage-specific expression of genes that support maturation, organization and mineralization of the bone extracellular matrix and developing bone-tissue-like nodules [1]. Furthermore, in the absence of mineralization, OP and OC are not induced at high level, whereas the expression of some bone cell phenotypic genes such as collagen or osteonectin remains unaffected.

A variety of osteoblastic markers, including alkaline phosphatase (ALP), OC and type I collagen, are stimulated by 1,25$(OH)_2D_3$ treatment in many osteoblastic cell lines including ROS 17/2.8 [11], MG-63, $SaOs_2$ and U_2Os cells [12,13]. Although 1,25$(OH)_2D_3$ has been shown to be a regulator of calcium binding proteins or calbindin-D, the functions of these molecules are poorly understood in bone cell cultures. Calbindin-Ds belong to the intracellular proteins which contain E-F hand calcium binding sites [14]. Calbindin-D28K (28 kDa calcium binding proteins) and calbindin-D9K (9 kDa calcium binding proteins) belong to this superfamily, each derived from distinct genes [15]. These proteins are distinguished from calmodulin and the other calcium binding proteins by their selective distribution [16] and requirements for vitamin D. Studies in recent years have suggested a regulation of calbindin-D28K expression and described tissue-specific regulation [17]. Calbindin-D28K is not only regulated by 1,25$(OH)_2D_3$ [18] but also by oestrogen, as indicated by studies in rat uterine tissue [19]. The close association between vitamin D induction of calcium transport and expression of calbindin-D28K in intestinal epithelial cells suggests a role for this protein in calcium transport [20]. Studies of calbindin-D28K expression have, until recently, been restricted to animal cells and non-osteogenic target cells. Two reports have described calbindin-D9K expression in rat osteoblast-like cells [21,22]. To date, information on expression of these proteins in human bone cells is limited [23] and their functions in matrix mineralization are unclear.

The aim of the current study was to investigate the synthesis and regulation of calbindin-D28K in HBMSC during matrix mineralization. To facilitate these studies, we developed an *in vitro* HBMSC culture model in which organization of the

Abbreviations used: HBMSC, human bone marrow stromal cells; ALP, alkaline phosphatase; OC, osteocalcin; OP, osteopontin; VDR, vitamin D receptor; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; IMDM, Iscove's modified Dulbecco's medium; FCS, fetal-calf serum; RT, reverse transcription.

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extracellular calcified matrix occured progressively from the first to the sixth subculture. Synthesis of calbindin-D28K in response to $1,25(OH)_2D_3$ was studied in cells arising from non-mineralized cultures and from the sixth subculture, which exhibited a calcified matrix and all of the osteoblastic markers, i.e. OC.

MATERIALS AND METHODS

Materials

Iscove's modified Dulbecco's medium (IMDM) and fetal-calf serum (FCS) were purchased from Gibco. Plastic culture dishes and Lab-Tek chamber slides were from Nunc (InterMed S.A., Paris, France). The bovine OC radioimmunoassay kit was purchased from Oris Industrie (Bagnols sur Ceze, France). Monoclonal antibodies specific to bovine osteonectin and OC were a gift from Dr. P. Seguin (Oris Industrie). Polyclonal antibodies specific for rat calbindin-D28K and calbindin-D9K, the corresponding cDN and $1,25(OH)_2D_3$ were kindly provided by Dr. M. Thomasset (INSERM U.458, Paris, France). Protein-A, isolated from *Staphylococcus aureus*, was purchased from Sigma. ^{125}I (100 mCi/ml) and $[^3H]1,25(OH)_2D_3$ (120 Ci/mmol) were purchased from CEA (Orsay, France) and Amersham International respectively. OP cDNA was generously provided by Professor G. Rodan (Merck-Sharpe Research Laboratories, West Point, PA, U.S.A).

Cell culture

HBMSC were prepared as described previously [24] with some modifications. In brief, human bone marrow aspirates were obtained from the femoral diaphysis of patients (aged 20–60 years) undergoing hip surgery after trauma. Bone marrow cells were separated by sequential passage through syringes fitted with 16, 18 or 21 gauge needles, and plated into 24-well plates (Nunc) in IMDM medium supplemented with 10% (v/v) FCS in a humidified atmosphere (5% CO_2/95% air at 37 °C). The culture medium was changed every 4 days. At confluency, approx. 3 weeks later, cells were detached with 0.2% (w/v) trypsin and 2 mM EDTA in 0.1 M PBS (pH 7.4) without Ca^{2+} and Mg^{2+}. For each subculture, three fractions were prepared for examination of cell maturation and differentiation; the first for cytochemical analysis of ALP activity, the second for quantitative analysis of ALP activity and OC synthesis and the third for subsequent subculture.

HBMSC response to $1,25(OH)_2D_3$ modulation of OC and calbindin-D28K synthesis was studied at the sixth subculture (mineralized cultures).

Intracellular ALP activity

Cells isolated from human bone marrow were seeded at 2×10^3 cells/cm^2 in Lab-Tek chamber slides and cultured for 3 days. ALP activity was determined cytochemically as described previously [25] using a diagnostic kit (85L-2, Sigma). Intracellular ALP activity was determined in cell lysates from confluent cultures as described by Majeska and Rodan [26]. The activity was expressed as nmol of P_i /30 min per μg of protein or per 10^5 cells.

OC synthesis

Cells were grown to confluency and the cell layer was scraped into 0.1 M PBS, pH 7.4. The cell suspension was sonicated at 20 kHz for 20 s, and the protein content determined using the Lowry method [27]. OC synthesis was measured using a specific radioimmunoassay kit (Oris Industrie) with an antibody against bovine OC. The detection limit for the assay was 1 ng/ml and data were expressed as ng of OC/μg of protein.

In situ hybridization

HBMSC arising from the sixth subculture were plated in 0.6 cm^2 dishes at 5×10^3 cells/cm^2. Cells were washed twice with 0.1 M PB, pH 7.4, containing 20 mM dithiothreitol and 5 mM $MgCl_2$, and fixed with freshly prepared 4% (v/v) paraformaldehyde in 0.1 M PBS, pH 7.4/5mM $MgCl_2$ for 20 min at room temperature. After washing, the cells were incubated for 30 min with proteinase K at 0.5 μg/ml (Boehringer-Mannheim) and acetylated using 0.1 M triethanolamine/0.25% acetic anhydride for 10 min in the dark. Cells were rinsed with $2\times$SSC ($1\times$SSC = 0.15 M NaCl/ 0.015 M sodium citrate), treated with 50% (v/v) formamide in $2\times$SSC for 10 min at 65 °C and prehybridized in $2\times$SSC/50% (v/v) formamide/10% (v/v) dextran sulphate/10% (v/v) Denhardt's solution containing 0.25 mg/ml of yeast tRNA and 0.5 mg/ml of denatured, sonicated salmon sperm DNA. Hybridization was performed with cDNA probes for rat calbindin-D28K and rat calbindin-D9K and a cDNA probe for rat OP labelled with digoxigenin using a digoxigenin-DNA-labelling kit (Boehringer-Mannheim). Approx. 200 ng of labelled probe diluted with hybridization buffer was added to each well and incubated overnight at 42 °C. After sequential washing with $2\times$SSC (twice), $1\times$SSC (twice), $0.5\times$SSC and $0.1\times$SSC, hybridized probes were revealed using an *in situ* hybridization detection kit (Boehringer-Mannheim). Controls included hybridization with a non-specific labelled DNA (Boehringer-Mannheim) and hybridization in the absence of a cDNA probe followed by incubation with or without anti-digoxigenin antibody.

Treatment of HBMSC with $1,25(OH)_2D_3$

HBMSC were seeded at 5×10^3 cells/cm^2 and grown to confluency. Confluent cultures were incubated for 24 h in serum-free IMDM followed by an incubation of 24 h, 48 h, 72 h or 96 h in IMDM containing 5% (v/v) charcoal-stripped FCS [18] in the presence of different concentrations of $1,25(OH)_2D_3$ (10^{-7} M, 10^{-8} M or 10^{-9} M) diluted with absolute ethanol. A similar volume of ethanol (vehicle) was added to the control cultures.

Examination of mineralization *in vitro* by ^{45}Ca uptake following $1,25(OH)_2D_3$ treatment

$1,25(OH)_2D_3$ modulation of mineralization was determined by the uptake of ^{45}Ca as described by Bellows et al. [29]. In brief, ^{45}Ca (0.2 μCi/ml) was added to differentiated HBMSC for the final 96 h of the culture period in the presence or absence of 10 mM β-glycerophosphate in IMDM containing 50 μg/ml of ascorbic acid and 5% (v/v) FCS. Thereafter, cultures were washed 4 times with 0.1 M PBS, pH 7.4, to remove unincorporated ^{45}Ca and the cell layer was scraped from the culture dish, collected in 500 μl of 0.1 M PBS, pH 7.4, and washed a further 3 times with PBS. The mineral phase was dissolved in 0.5 ml of 10% (v/v) formic acid for 24 h and the entire sample was transferred to scintillation vials containing 5 ml of Instagel (Packard) and counted by scintillation spectroscopy. A parallel study was carried out using cells treated with $1,25(OH)_2D_3$ (10^{-8} M) for 72 h. Results were expressed as c.p.m. $\times10^{-3}/10^6$ cells.

Radioimmunodetection of cellular proteins

Total cellular proteins were obtained from cell lysates and 1 μg was applied to nitrocellulose membranes (HAWP 304 FO,

Schleicher and Schuell). Non-specific sites were saturated with 0.1 M PBS, pH 7.4, containing 3% (w/v) BSA at 37 °C for 2 h. After washing with 0.1 M PBS, pH 7.4, containing 0.5% (v/v) Tween 20 and 0.5% (w/v) BSA (PBS–Tween-buffer), the blots were incubated overnight at 26 °C in the same buffer with monoclonal anti-osteonectin and anti-OC (1:1000) and polyclonal anti-calbindin-D28K and -D9K (1:100) antibodies. Incubation with rabbit anti-mouse immunoglobulin serum diluted in PBS-Tween buffer at 1:1000 for 6 h was added before Protein A detection. After washing in PBS-Tween buffer, fixed immunoglobulins were revealed by ^{125}I-labelled Protein A (1.8×10^6 c.p.m./μg), which was labelled using the chloramine T method [30]. Filters were washed using 0.1 M PBS, pH 7.4, containing 0.4% (w/v) sarcosyl and 0.5% (v/v) Tween 20 and were exposed on MP film (Amersham) with intensifying screens for 24 h at −80 °C.

Reverse transcription (RT)-PCR analysis

Cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) for 24 h as described previously. Total RNA was extracted using RNeasy total RNA kit (Qiagen) and 2 μg was used as template for cDNA synthesis with a superscript preamplification system (Gibco-BRL) in 20 μl (final volume) containing 20 mM Tris/HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 μg/μl BSA, 10 mM dithiothreitol and 0.5 mM each of dATP, dCTP, dGTP, dTTP, 0.5 μg of oligo$(dT)_{12-18}$ and 200 units of reverse transcriptase. After an incubation of 50 min at 42 °C, the reaction was stopped by increasing the temperature to 70 °C for 15 min; the samples were then kept on ice. Synthesized cDNA were treated with 2 units of RNase H (*Escherichia coli*) at 37 °C for 20 min and samples were mixed with PCR cocktail (20 mM Tris/HCl, pH 8.4/50 mM KCl/2.5 mM $MgCl_2$ containing 0.1 μg/μl BSA, 0.1 mM each of dATP, dCTP, dGTP, dTTP and 2.5 units of Taq polymerase) and 0.5 μM each of forward and reverse primers (5′-CCCCGGAT-CTGTGGGGTGTGT-3′ and 5′-CTCCTCATGCAAGTTCA-GCTT-3′) in 100 μl final volume. Amplification was performed in a Hybaid thermocycler using the following conditions: denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 10 s, 55 °C for 30 s and 72 °C for 1 min. PCR products were electrophoretically separated on a 1% agarose gel containing ethidium bromide.

RESULTS

Cell culture and phenotypic expression

Quantitative measures of bone-related markers performed on the different subcultures of HBMSC exhibit a significant difference of ALP activity and OC content (Figure 1). Cells from the first to third subculture exhibited low ALP activity (15 ± 0.5 nmol P_i/30 min per 10^5 cells, mean ± S.D.) and synthesized trace amounts of OC. In contrast, cells from the sixth subculture exhibited high ALP activity (28 ± 1.5 nmol P_i/30 min per 10^5 cells, mean ± S.D.) and synthetized OC (0.054 ng/μg of protein), a specific marker of mature osteoblasts. Bone nodules were observed only in the sixth subculture (Figure 2A), which also contained high ALP activity (Figure 2B) and Von Kossa staining, indicative of nodules containing high levels of calcium (Figure 2C). In these cultures, ALP activity and OC synthesis remained unchanged until the tenth subculture after which both parameters decreased. Cells isolated from the sixth passage (differentiated HBMSC) were used to measure $1,25(OH)_2D_3$ effects. *In situ* hybridization analysis of differentiated HBMSC demonstrated OP expression (Figure 3A), another bone matrix protein. These cultures were also found to express calbindin-D28K mRNA

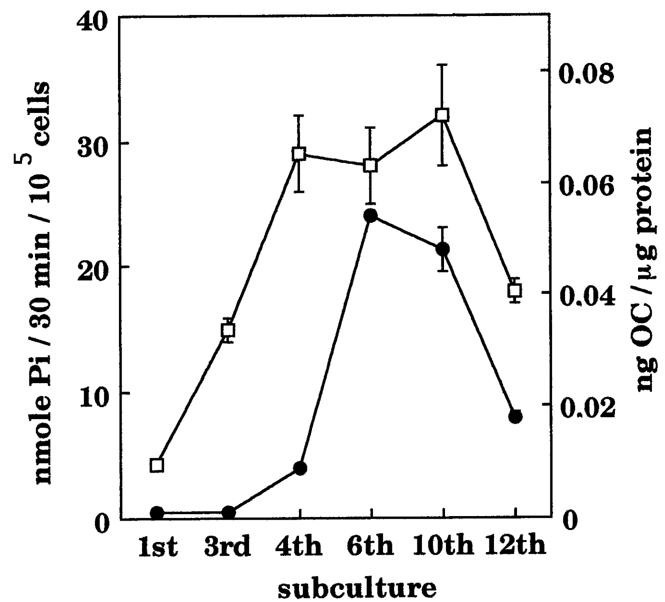

Figure 1 Biochemical analysis of ALP activity and OC synthesis in HBMSC derived from different subcultures

ALP activity (□) was measured according to Majeska and Rodan [26] and expressed in nmol P_i/30 min per 10^5 cells. OC synthesis in the cell layer (●) was measured by radioimmunoassay as described in the Materials and methods section and the results are expressed as ng of OC per μg of total cellular protein quantified by the Lowry method [27]. Values were expressed as means ± S.D. ($n = 4$).

(Figure 3B), although no specific reaction was obtained with the calbindin-D9K cDNA probe (Figure 3C). Table 1 summarizes the different protein synthesis or gene expression in relation to the subculture number (third or sixth subculture). As described previously [24], type I collagen and osteonectin were also synthesized by cultures arising from the subculture, whereas in cells arising from the third subculture, only type I collagen synthesis and a low ALP activity was detected. However, by using RT-PC, dot-blot or *in situ* hybridization, we demonstrated that cells arising from the third subculture expressed mRNA for ALP and OC (results not shown).

VDR expression

Dose- and time-course studies with $1,25(OH)_2D_3$ in differentiated HBMSC indicated maximal stimulation of ALP activity, and OC synthesis was observed at 10^{-8} M following 24 h exposure to $1,25(OH)_2D_3$ (results not shown). As shown in Figure 4, RT-PCR analysis using total RNA extracted from cells (sixth subculture) revealed a 925 bp band corresponding to the PCR product amplified from human VDR cDNA.

Effect of $1,25(OH)_2D_3$ on calcium uptake into the matrix layer

In mineralized cultures (sixth subculture), $1,25(OH)_2D_3$ treatment induced a two-fold stimulation of ^{45}Ca accumulation into the matrix after 96 h of treatment, when compared with cells treated only with β-glycerophosphate and ascorbic acid (Figure 5).

Effect of $1,25(OH)_2D_3$ on the calbindin-D28K protein synthesis

Total cellular proteins were extracted from treated ($1,25(OH)_2D_3$, 10^{-8} M for 24 h) and untreated HBMSC arising from the third

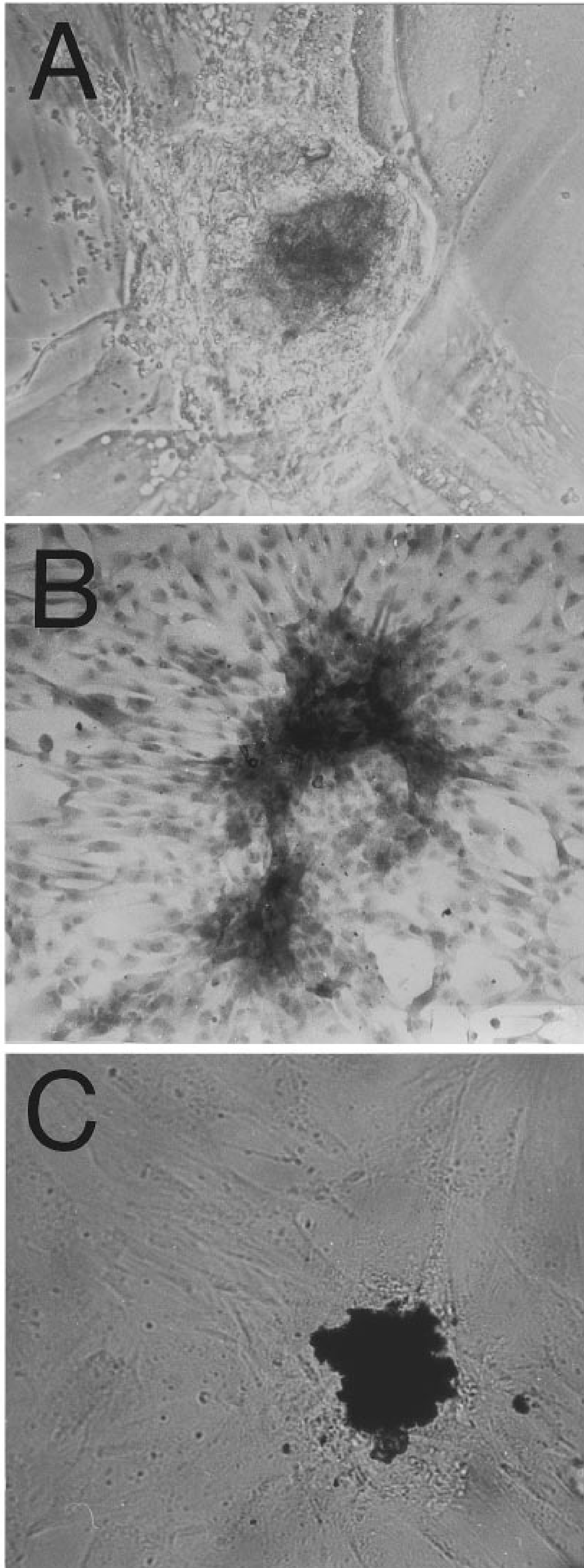

Figure 2 Cell nodule formation in differentiated cells arising from the sixth subculture

(**A**) Mineralization of nodules (magnification $\times$ 9). (**B**) ALP activity of the nodules (magnification $\times$ 18). (**C**) Von Kossa staining of nodules (magnification $\times$ 9).

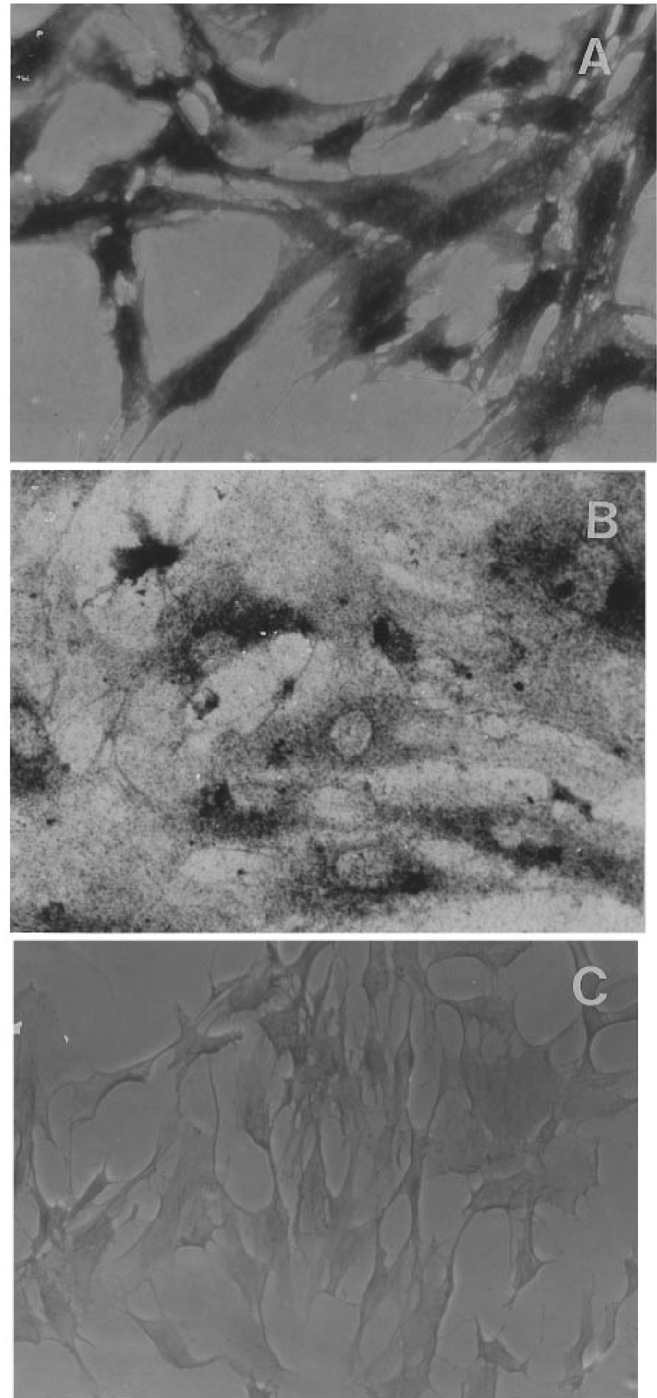

Figure 3 *In situ* hybridization in differentiated HBMSC

Differentiated HBMSC (sixth subculture) were seeded in Lab-Tek chamber slides (0.6 cm^2) at 5×10^3 cells/cm^2 and fixed with 4% (v/v) paraformaldehyde. After various treatments, as described in the Materials and methods section, cells were hybridized overnight at 42 °C with cDNA probes labelled with digoxigenin (200 ng) diluted in the hybridization buffer. (**A**) OP (magnification $\times$ 40). (**B**) calbindin-D28K (magnification $\times$ 40). (**C**) calbindin-D9K (magnification $\times$ 10). Hybridized probes were revealed using a detection kit (Boehringer Mannheim).

and sixth subculture and the synthesis of calbindin-D28K and -D9K was followed by dot-immunodetection using polyclonal antibodies against calbindin-D28K and -D9K. For these semi-quantitative assays, the same amount of cellular proteins (1 μg) was applied to the nitrocellulose membrane. In mineralized cultures (sixth subculture), $1,25(OH)_2D_3$ treatment induced 1.7-

Table 1 Gene expression and protein synthesis related to HBMSC subculture

Protein synthesis was identified by cytochemical analysis (ALP), radioimmunoassay (OC), dot-immunodetection (calbindin-D28K, osteonectin) or by using [^{3}H]proline incorporation to detect type I collagen synthesis. mRNA expression was detected by RT-PCR or *in situ* hybridization (HI) or dot-blot. Key: [−], no synthesis; [±], weak synthesis; [++], moderate synthesis; [+++], high synthesis.

	Unmineralized matrix (third subculture)	Mineralized matrix (sixth subculture)
Protein synthesis	ALP [±] OC [−] Type I collagen [+]	ALP [+++] OC [+++] Type I collagen [+++] Osteonectin [++] Calbindin-D28K [++]
Gene expression	ALP (RT-PCR) OC (RT-PCR) OP (HI, dot-blot) Calbindin-D28K (HI, dot-blot) OP (HI, dot-blot) (results not shown)	ALP (RT-PCR) (results not shown) OC (RT-PCR) (results not shown) OP (HI, dot-blot) Calbindin-D28K (HI, dot-blot) OP (HI, dot-blot)

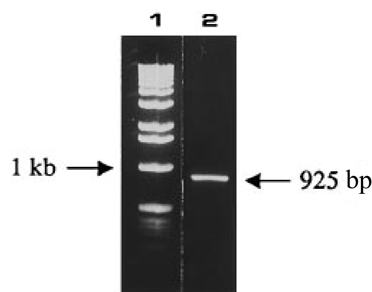

Figure 4 Expression of human VDR in differentiated HBMSC

Total RNA was extracted from differentiated HBMSC (sixth subculture) and 2 μg was used for RT-PCR performed with the two primers obtained from a human VDR sequence. The PCR product was analysed by electrophoresis on a 1% agarose gel. Lane 1, DNA marker (1 kb ladder, Gibco-BRL); Lane 2, amplified VDR cDNA.

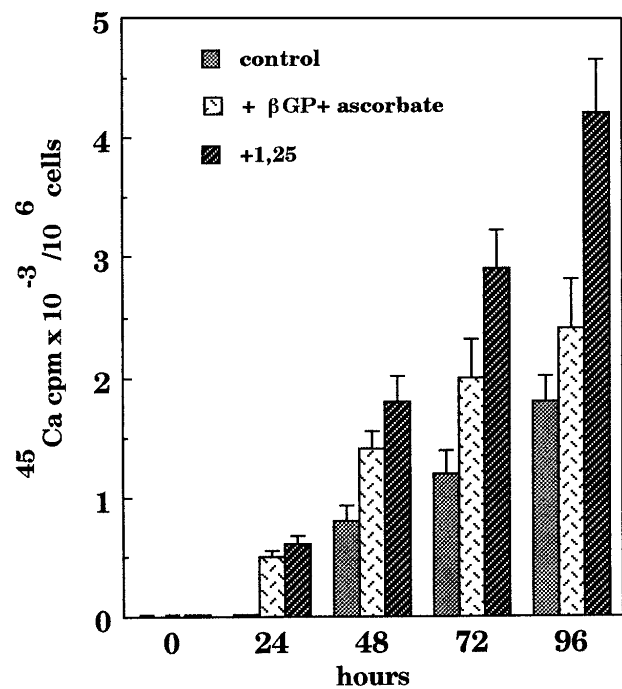

Figure 5 ^{45}Ca uptake in differentiated HBMSC

Cells were treated with fresh medium containing ^{45}Ca at 0.2 μCi/ml and supplemented or not with 10 mM β-glycerophosphate (β-GP) and 50 μg/ml ascorbic acid (ascorbate). A similar experiment was performed using cells treated with $1,25(OH)_2D_3$ (1,25) for 72 h. At each time point, cell number was estimated and ^{45}Ca uptake in the cell layer was determined as described in the Materials and methods section. Values are expressed as means ± S.D. ($n = 4$).

fold stimulation of the calbindin-D28K synthesis (Figure 6A) compared with untreated cells. No cross-reactivity was observed with anti-calbindin-D9K antibody with control or $1,25(OH)_2D_3$-treated cells (Figure 6B). Vitamin D_3 stimulated OC (positive control) synthesis 1.4-fold (Figure 6C) while osteonectin (negative control) was unaffected by $1,25(OH)_2D_3$ treatment (Figure 6D). In contrast, in non-mineralized cultures, as for ALP activity or OC synthesis, calbindin-D28K synthesis was undetectable even in the presence of $1,25(OH)_2D_3$ (results not shown).

DISCUSSION

In vitro progressive development of osteoblast phenotype has been shown to follow a co-ordinate and temporal sequence: proliferation, extracellular matrix maturation and mineralization [31,32]. This human bone marrow cell culture model provided an ideal means of obtaining a homogeneous population of mature osteoblasts after progressive subcultures. These human differentiated cells were not modified by any stimulating treatment such as dexamethasone, generally used in osteoblast culture from bone marrow, and more interestingly, could be mineralized *in vitro* in the presence of β-glycophosphate and ascorbic acid, and the presence of $1,25(OH)_2D_3$ increased this process. Bone protein synthesis related to the subculture was well defined, and could be connected to the matrix mineralization process. ALP activity appeared during the third subculture, whereas OC content was detectable only in cells arising from the fourth subculture. HBMSC from the sixth subculture then produced an extracellular matrix committed to the bone cell differentiation process (type I

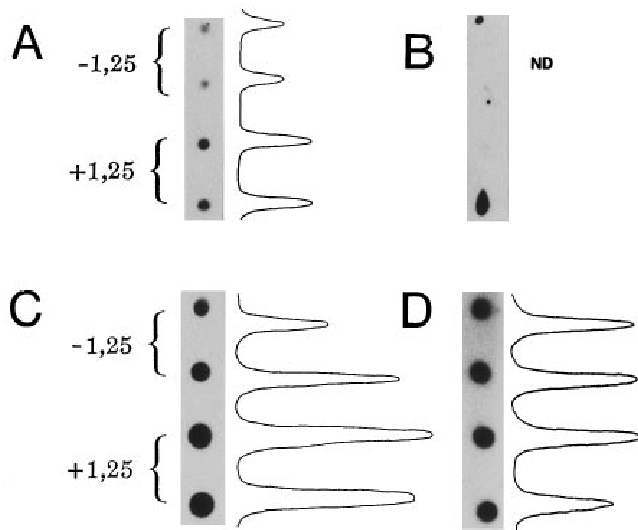

Figure 6 Dot-immunodetection of cellular proteins extracted from differentiated HBMSC (sixth subculture) treated with $1,25(OH)_2D_3$

Confluent cells were treated with 10^{-8} M $1,25(OH)_2D_3$ for 24 h. Total cellular protein (1 μg) extracted from untreated (−1,25) and treated cells (+1,25) was dot-blotted in duplicate on to nitrocellulose membrane and incubated overnight at 26 °C with polyclonal antibodies (diluted 1:100) raised against (**A**) calbindin-D28K, (**B**) calbindin-D9K or monoclonal antibodies (diluted 1:1000) against (**C**) OC or (**D**) osteonectin. Fixed immunoglobulins were revealed by incubation with ^{125}I-labelled Protein A (0.5×10^6 c.p.m./ml) and exposure of the membrane to MP film and intensifying screens for 24 h at −80 °C. The intensity of the reaction was measured by ultroscan spectrophotometry (LKB). ND, not determined.

collagen, osteonectin, OC, OP and calbindin-D28K). With regard to matrix mineralization, this was never observed in undifferentiated cells arising from the third subculture. In contrast, cells arising from the sixth subculture (differentiated HBMSC) produced a specific bone mineralized matrix constituted by OC, OP-associated bone nodule formation, and shown by von Kossa staining to be loaded with calcium phosphate. After the tenth subculture, the decrease in OC synthesis and in ALP activity suggested a loss of osteoblast phenotype, which is in agreement with previous studies *in vitro* on human osteoblast cells [33,34].

The well-known hormonal response of bone cells to $1,25(OH)_2D_3$ was characterized, in these HBMSC, by the detection of VDR mRNA.

Vitamin D regulation of type I collagen, OC or ALP activity are well studied in bone cells. To date, calbindin-D28K expression and synthesis in human bone cells has not been widely studied [23] and their association with a specific stage of bone tissue development has not been defined [16]. The methods of investigation used in the present work (dot-immunodetection and *in situ* hybridization) allowed identification, for the first time, of the expression and synthesis of calbindin-D28K in HBMSC. Results were obtained either with antibodies raised against the rat calbindin-D28K, which were observed to cross-react with human protein [35], or with the rat calbindin-D28K cDNA, which could hybridize with the human calbindin-D28K mRNA [35]. Dot-immunodetection performed on differentiated HBMSC confirmed the synthesis of calbindin-D28K and addition of 1,25-$(OH)_2D_3$ enhanced its synthesis and that of other osteoblastic markers, e.g. ALP or other bone matrix proteins involved in the mineralization process such as OC. However, calbindin-D28K (like OC) was not detected in non-mineralized cultures using immunological probes, even after treatment with vitamin D_3. Thus, this study provides the first evidence of a link between calbindin-D28K expression and osteoblast differentiation states. We demonstrated that only differentiated HBMSC, implicated in the matrix mineralization process, synthesize calbindin-D28K. In addition, $1,25(OH)_2D_3$ treatment induced an increase in calbindin-D28K synthesis, in parallel with bone-related proteins or calcium uptake into the matrix.

Antibodies against rat calbindin-D9K showed no reaction with cellular proteins isolated from cells of the sixth subculture, probably attributable to the absence of cross-reactivity with the human protein. As previously described by Balmain [36], using Northern-blot analysis, human calbindin-D9K mRNA can be analysed using a rat cDNA probe. However, in the present studies, using *in situ* hybridization or dot-immunodetection, no specific response was observed compared with the control. This inability to detect calbindin-D9K could be due to a lower expression in bone cells compared with the expression of calbindin-D28K. As reported by Berdal et al. [16], the higher level of calbindin-D28K compared with calbindin-D9K may be a common feature of bone tissues.

Our model appears to mimic closely the progressive differentiation process, in the absence of added glucocorticoids to the culture. The induction of the osteoblastic phenotype is an important step and all synthesized proteins should play a role in extra-cellular matrix production and mineralization. The mineralization process is essential for bone formation and, at present, only a few proteins have been assumed to act on extracellular matrix calcification (e.g. OC, OP, ALP). The close correlation with calbindin-D28K and OC synthesis and the regulation of their synthesis by $1,25(OH)_2D_3$, which, in addition, modulated the calcium uptake into the matrix, suggest a general function of calbindin-D in calcium mobilization in mineralized cultures [16]. As suggested by Berdal et al. [16], calbindin-D may contribute to the characterization of the terminal differentiation of osteoblasts, odontoblasts and ameloblasts in parallel with other matrix proteins. Our present study supports the concept that calbindin-D28K, induced by $1,25(OH)_2D_3$, may be involved in the elaboration of a mineralized matrix, and is confirmed by the calcium uptake observed following the addition of vitamin D_3. This matrix could trap calbindin-D28K stocked calcium in order to induce or increase the mineralization process. However, it is important to note that not all of the calcium binding proteins behave similarly in the mineralization event. For example, another calcium binding protein, calreticulin [37,38], has been shown to inhibit the mineralization process and is correlated with the absence of mineralization nodules in long-term cultures. This inhibition appears to be related to the inhibition of vitamin D-induced expression of OC and $1,25(OH)_2D_3$-induced ^{45}Ca accumulation [38] .

In conclusion, these results identify and characterize the genomic actions of $1,25(OH)_2D_3$ in HBMSC, mediated by the VDR, the presence of which was indicated by RT-PCR detection of mRNA. Vitamin D_3, an osteotropic agent, stimulated not only OC and ALP activity but stimulated calbindin-D28K synthesis in mineralized cultures. These new findings *in vitro* suggest the transport of calcium in bone cells as a role for calbindin-D28K in the mineralization process. Furthermore, calbindin-D28K may play a direct role in calcium storage, when trapped within the bone matrix. To further investigate the physiological role of calbindin-D28K, mice have been generated by targetted gene disruption [39]. The absence of calbindin-D28K production increased intracellular calcium concentration when compared with wild mice. Calbindin-D28K knockout mice provide direct evidence that calbindin acts as a calcium buffer and as an important modulator of induced intracellular calcium transients

[39]. However, no studies have been done on the mineralization process in these knockout mice. Futher studies are in progress to elucidate mechanisms and factors controlling calbindin-D28K expression in human bone cells.

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